UNIVERSITI PUTRA MALAYSIA

SEQUENCE AND FUNCTIONAL ANALYSES OF SALINITY TOLERANCE GENES ISOLATED FROM THE MANGROVE PLANT, ACANTHUS EBRACTEATUS (SEA HOLLY)

NGUYEN PHUOC DANG.

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By

NGUYEN PHUOC DANG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chairman : Ho Chai Ling, PhD

Faculty : Biotechnology and Biomolecular Sciences

Salinity is a major abiotic stress that greatly affects plant growth and crop production. Most trees and crop plants are sensitive to salty conditions. Sodium ions are toxic to plants because of their adverse effects on potassium nutrition, cytosolic enzymes activities, photosynthesis and metabolism. Mangrove plants are good models to study plant tolerance to salinity as they possess salinity tolerance genes that allow them to survive under with high salinity conditions. The objectives of this study are to identify, isolate and characterize salinity tolerance genes from a mangrove plant, *Acanthus ebracteatus* using expressed sequence tag (EST) and bacterial functional assay approaches.

The leaves of *A. ebracteatus* were collected from the mangrove area at Morib, Selangor. Total RNA was isolated from the leaves of *A. ebracteatus,* and a cDNA library was constructed from cDNA fractionated between 500 to 5,000 bp. A total of eight hundred sixty four randomly selected clones were

isolated from the primary cDNA library from which 521 clones were sequenced. Among these ESTs, 138 of them were assembled into 43 contigs whereas 383 were singletons. A total of 349 of these ESTs showed significant homology to functional proteins and 18 % of them are particularly interesting as they correspond to genes involved in the stress response. Some of these clones, including mannitol dehydrogenase, plastidic aldolase, secretory peroxidase, ascorbate peroxidase, and vacuolar H^+-ATPase, may be related to salinity tolerance mechanisms such as osmotic homeostasis, ionic homeostasis and detoxification.

In this study, a bacterial functional assay was also performed to identify cDNAs that confer salinity tolerance. A total of 120 salinity tolerant candidate genes from *A. ebracteatus* were isolated from 2 X YT medium supplemented with 400 mM NaCl and sequenced. Among these clones, 27 of them may be related to salinity tolerance such as manganese superoxide dismutase (Mn-SOD), putative salt tolerance protein, glutathione S-transferase, etc. The results showed that plants and bacteria may share some similar mechanisms for salinity tolerance.

A total of six cDNA clones from *A. ebracteatus* were fully sequenced and three of them were characterized by Southern hybridization and Northern hybridization. Clone A290 encoded a putative plastidic aldolase that may be involved in osmoprotection by converting triose phosphate into hexose. This gene was found to be expressed predominantly in the leaves of *A. ebracteatus*. There may be more than one family member of plastidic

aldolase in *A. ebracteatus*. Meanwhile, clone A303 was found to be a putative H^+-ATPase, an enzyme known to play an important role in ion homeostasis, a salinity tolerance mechanism. This gene most probably exists as a single copy gene in *A. ebracteatus*. The expression of H^+-ATPase was detected in all tissues of *A. ebracteatus*. Clone A325 encoded a putative monodehydroascorbate reductase which is involved in the detoxification mechanism. This gene was also expressed in all tissues and is most probably a single copy gene in the genome of *A. ebracteatus*.

Sequence analysis of the putative salinity tolerant cDNAs isolated by bacterial functional assay and ESTs suggested that the salinity tolerance mechanisms in *A. ebracteatus* may involve ion homeostasis, osmotic homeostasis, detoxification and other supporting mechanisms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

ANALISIS JUJUKAN DAN FUNGSI GEN-GEN KETAHANAN TERHADAP GARAM DARIPADA TUMBUHAN BAKAU, *Acanthus ebracteatus* (SEA HOLLY).

Oleh

NGUYEN PHUOC DANG

Desember 2005

Pengerusi : Ho Chai Ling, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Kegaraman (salinity) merupakan tekanan abiotik utama yang amat berkesan terhadap pertumbuhan pokok dan penghasilan tanaman. Kebanyakan pokok dan tanam-tanaman adalah sensitif terhadap keadaan yang masin. Ion natrium adalah toksik kepada tumbuhan disebabkan kesan yang buruk terhadap nutrisi potassium, aktiviti enzim sitosolik, fotosintesis dan metabolisma. Pokok bakau merupakan model yang baik untuk mengkaji ketahanan tumbuhan terhadap garam kerana mereka mempunyai gen ketahanan terhadap garam yang membolehkannya hidup di dalam kegaraman yang tingyi. Tujuan kajian ini adalah untuk mengenalpasti, memencilkan dan mencirikan gen ketahanan terhadap garam daripada pokok bakau, *Acanthus ebracteatus* dengan menggunakan 'expressed sequence tag' (EST) dan pendekatan esei bakteria berfungsi.

Daun *A. ebracteatus* telah dikumpulkan daripada kawasan hutan bakau di Morib, Selangor. RNA telah diekstrak daripada daun *A. ebracteatus* dan

perpustakaan cDNA telah dibina daripada fraksi cDNA di antara 500 ke 5000 bp. Sejumlah 864 klon telah dipilih secara rawak dan dipencilkan daripada perpustakaan cDNA primer di mana 521 klon telah dijuzuk. Di antara EST tersebut, 138 daripadanya wujud sebagai 43 kontig dan 383 yang selebihnya adalah singleton. Sebanyak 349 daripada EST ini menunjukkan homologi yang berkesan terhadap protein yang berfungsi dan 18 % daripadanya amat menarik kerana berhubungkait dengan gen-gen yang terlibat dengan tindak balas tekanan. Di antara klon-klon ini termasuk mannitol dehidrogenase, plastidik adolase, sekretori peroksidase, askorbat peroksidase, vakuolar H^+-ATPase, yang mungkin berhubungkait dengan homeostasis osmotik, homeostasis ionik dan detoksifikasi.

Di dalam kajian ini, esei bakteria berfungsi dibuat untuk memencilkan cDNA yang mempunyai ketahanan terhadap garam. Sebanyak 120 gen yang menunjukkan ketahanan terhadap garam daripada *A. ebracteatus* telah dipencilkan daripada media 2 X YT yang mengandungi 400 mM NaCl dan analisis jujukan telah dibuat. Antara klon-klon ini, 127 daripadanya mungkin berhubungkait dengan protein ketahanan garam, glutathione S-transferase dan sebagainya. Keputusan menunjukkan bahawa tumbuhan dan bakteria berkongsi sesetengah mekanisma yang serupa di dalam ketahanan terhadap garam.

Sebanyak enam cDNA klon daripada *A. ebracteatus* telah jujuk sepenuhnya dan tiga klon cDNA telah dicirikan dengan menggunakan penghibridan 'Southern' dan 'Northern'. Klon A290 yang mengkodkan plastidik adolase,

mungkin terlibat di dalam 'osmoprotection' dengan mengubahkan trios fosfat kepada heksosa. Gen ini dizahirkan dengan banyaknya pada daun *A. ebracteatus*, dan didapati mungkin lebih daripada satu salinan dalam *A. ebracteatus* yang tergolong dalam keluarga yang sama. Manakala klon A303 merupakan putatif H^+-ATPase, yang memainkan peranan penting di dalam homeostasis ion di dalam mekanisma toleransi terhadap garam. Gen ini berkemungkinan besar wujud sebagai satu salinan. Pengzahiran H^+-ATPase telah dikesan pada semua tisu *A. ebracteatus*. Klon A325 yang mengekodkan putatif monodehidroaskorbat reduktase mungkin terlibat mekanisma nyah-toksik. Gen ini juga dizahirkan di dalam semua tisu dan berkemungkinan besar adalah salinan gen tunggal di dalam genom *A. ebractreatus*.

Analisa jujukan putatif cDNA yang berketahanan terhadap garam yang dipencilkan melalui esei bakteria berfungsi dan EST mencadangkan bahawa mekanisma ketahanan terhadap garam di dalan *A. ebracteatus* mungkin melibatkan hemoestasis ion, homeostasis osmotik, detoksifikasi dan mekanisma sokongan yang lain.

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I certify that an Examination Committee met on 14th December 2005 to conduct the final examination of Nguyen Phuoc Dang on his Doctor of Philosophy thesis entitled "Sequence and Functional Analyses of Salinity Tolerant Genes Isolated from the Mangrove Plant, *Acanthus ebractearus* (sea holly)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Maziah Mahmood, PhD
Professor
Faculty of Bitechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Siti Nor Akhmar Abdullah, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Norihan Mohd. Saleh, PhD
Associate Professor
Faculty of Bitechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Helen Nair, PhD
Professor
Faculty of Applied Sciences
Asian Institute of Medicine, Science and Technology
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 JAN 2006

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Ho Chai Ling, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Jennifer Ann Harikrishna, PhD
Associate Professor
Malaysia University of Science and Technology
(Member)

Raha Abdul Rahim, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/ Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

07 FEB 2006

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

NGUYEN PHUOC DANG

Date: 17 Jan 2006

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α	alpha
β	beta
λ	lambda
×g	gravitational acceleration
μg	microgram
μL	microliter
°C	degree Centigrade
%	percentage
AMV	avian myeloblastosis virus
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
Ca	calcium
cDNA	complementary DNA
CIP	calf intestinal phosphatase
Cl	chloride
cm	centimeter
CsCl	cesium cholride
CTAB	hexacetyltrimethyl ammonium bromide
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxy-guanosine-5'-triphosphate

DMSO	dimethylsulphonyl oxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotides
ds	double-stranded
DTT	dithiothreitol
dTTP	thymidine-5'-tryphosphate
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactoside
K	potassium
kb	kilo base-pair
L	liter
LB	Luria-bertani
LiCl	lithium chloride
M	molar
Mg	magnesium
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MOPS	3-(N-morpholino) propane-sulphonic acid
mL	milliliter
mM	millimolar

mRNA	messenger RNA
Na	sodium
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	nanogram
NH_4OAc	ammonium acetate
OD	optical density
ORF	open reading frame
PCR	polymerase chain reactions
PVP	polyvinylpyrrolidone
pfu	plaque forming units
ppm	part per million
RNA	ribonucleic acid
RNase A	ribonuclease A
ROS	reactive oxygen species
rpm	revolution per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SOS	salt overly sensitive
TAE	tris acetate EDTA
TE	Tris-EDTA
U	unit
v/v	volume per volume

w/v	weight per volume
X-gal	5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Salinity is one of the major abiotic stresses that affects plant growth and productivity globally. Salt stress can lead to changes in development, growth and productivity, and severe stress may threaten survival. High salinity causes both hyper osmotic and hyper ionic stress effects, and the consequence of these can be lethal to the plants. Therefore, a better understanding of the mechanisms that enable plants to adapt to salinity stress and to maintain growth will ultimately help in selection of stress tolerant cultivars for planting in saline soil.

In addition, due to the increased demand for food crops and plant products, the use of irrigated agriculture in the world has increased during the past 35 years (Chaturvedi, 2000). The rapid expansion in irrigation combined with the increase use waters containing high salt have led to the decrease in crop productivity, which is primarily due to salinity stress.

Mangroves represent the dominant soft bottom plant communities of the marine-terrestrial transition in tropical and subtropical regions. The mangrove species are members of terrestrial families that have adaptations to survive under conditions of high salinity, low oxygen and nutrient availability in the soil (Pernetta, 1993). Mangroves are divided into two distinct groups on the basis of their salt management strategies. One is “secreters” which have salt

glands or salt hairs and the other is "non secreters" lacking such morphological features for excretion of excess salt. The *Acanthus ebracteatus* is included in the first group.

Acanthus. spp is also known as Sea holly, holly mangrove, and "jeruju putih" (Malay). *A. ebracteatus* grows on the mud near the tide mark, often on mud lobsters mound. It can grow equally well under trees and in open areas, the plant can sometimes cover a large area and form thickets. The most striking feature of mangrove plant species is their ability to tolerate NaCl found in seawater up to the 500 mM level (Takemura *et al.*, 2000).

In order to elucidate salt tolerance mechanisms in higher plants, numerous key factors have been cloned such as late embryogenesis abundant protein (LEA) (Xu *et al.*, 1996), P5CS (Kishor *et al.*, 1995), DREB1A (Kasuga *et al.*, 1999), and AtNHX1 (Apse *et al.*, 1999). In contrast, the mechanisms that explain how plants can grow in saline conditions are still unclear. In order to grow under salinity stress, the mangrove plants must have acquired some proteins essential for salt tolerance mechanisms during their evolution. Recently, many reports addressing the mechanisms of mangrove plants at organ level were available (Werner and Stelzer, 1990). However, there were few reports about their mechanisms at molecular level because only a few model systems are available to analyze these mechanisms (Yamada *et al.*, 2002). Therefore, it is necessary to carry out molecular genetic studies on mangrove.